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The Rejection of Claims 13, 16, and 18-22 under 35 U.S.C. §112, first paragraph

Claims 13, 16, and 18-22 stand rejected as not enabled by the specification. This rejection is respectfully traversed.

There are two grounds for rejecting the claims. First, the reference to Gen Bank Accession Number X76534 at page 26 is said to be insufficient as the sequence is said to be essential material and the GenBank data change over time. Applicants have amended the specification to include the actual sequence of X76534 as SEQ ID NO:17. A declaration of Dr. Riggins (Tab A) supports this amendment.

Second, the methods of the claims are said not to be enabled because they require undue experimentation to practice. This assertion is based on the specification's provision of prophetic rather than working examples. This basis for rejection is, however, legally insufficient. "An application need not contain an example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation." M.P.E.P. § 2164.02, citing *In re Borkowski*, 422 F.2d 904, 908 (C.C.P.A. 1970).

The Federal Circuit enumerated eight factors which may be considered in determining whether a specification is enabling. The presence or absence of working examples is just one of these factors. The eight factors are:

(1) the quantity of experimentation necessary;
(2) the amount of direction or guidance provided;
(3) the presence or absence of working examples;
(4) the nature of the invention;
(5) the state of prior art;
(6) the relative skill of those in the art;
(7) the predictability or unpredictability of the art; and
(8) the breadth of the claims.

In re Wands, 858 F.2d. 731, 737 (Fed. Cir. 1988). If one also considers factors 4, 5, 6, and 8, the enablement of the claims is apparent.

The Breadth of the Claims (Factor 8)

The claims are directed to the delivery of a reagent to a particular type of brain tumor, a glioblastoma. The delivery is targeted by an antibody which binds to a particular portion of a particular protein, *i.e.*, the extracellular domain of GPNMB. Thus, the breadth of the claims is not large in these aspects.

The Relative Skill of Those in the Art (Factor 6)

Those of skill in the art of brain analysis and treatment are among the most highly educated and skilled professionals in our society. Typically these workers have one or more post-baccalaureate degrees with many additional years of training and research beyond.

The State of Prior Art

The prior art contains many examples of antibodies which have been used to target reagents to tumors.

Zevalin™ is an antibody specific for CD20 antigen which is conjugated to a linker-chelator which binds to Indium-111 or Ytrium-90. Zevalin™ is currently in clinical use for treating certain cancers. (Tab B).

ProstaScint™ is an antibody which is conjugated to an Indium-111 label. The antibody recognizes a prostate specific membrane glycoprotein. ProstaScint™ is in clinical use for tomographic imaging. (Tab C).

Bexxar™ is an iodine-131 labeled antibody which is used clinically to treat relapsing lymphoma. (Tab D).

Mylotarg™ is an FDA approved therapy for fighting relapsed acute myeloid leukemia. Mylotarg ™ is an antibody drug conjugate that targets chemotherapy to CD33 antigen on leukemia cells. (Tab E).

A single chain for antibody for EGFRvIII, an antigen on glioblastoma, breast, ovary, and lung cancers, was genetically fused to *Pseudomonas* exotoxin A. The immunotoxin was completely stable in human serum at 37°C for 24 hours. (Tab F).

Antibody B3 recognizes an epitope on tumors that invade the intrathecal space and cause neoplastic meningitis. This antibody was used to construct a single chain antibody genetically fused to *Pseudomonas* toxin PE38. The antibody toxic fusion significantly extended survival in an animal model and some survivors were considered histologically cured. (Tab G).

U.S. Patent No. 6,306,393 teaches the administration of a therapeutic protein which is a radio-labeled anti-CD19 immunoconjugate. See claim 7. Radiolabeled anti-CD20, anti-CD52, or anti-CD74 immunoconjugates are also taught. These are administered to treat a B-cell malignancy. (Tab H).

U.S. Patent No. 5,872,223 teaches an immunocongugate of an enzyme inhibitor covalently bound to an antibody. The inhibitor induces apoptotic cell death in target cells. Col. 2, lines 51-52. The immunoconjugates kill target cells *in vivo* in animal models. Col. 3, lines 1-8. (Tab I).

U.S. Patent No. 5,601,825 teaches drugs or toxins conjugated to antibody molecules. The immunoconjugates are useful for treating leukemias and lymphomas (Col. 2, lines 50-52) and tumors (claims 1, 10). (Tab J).

U.S. Patent No. 6,146,628 teaches fusions of a targeting moiety, such as a monoclonal antibody to CD2, CD3, CD4, CD5, CD7, CD13, CD19, CD22, CD24, CD33, CD40, CD45, and CD72 antigen and a toxin Pokeweed Anti-viral Protein (PAP). Claims 1 and 6. These are used to treat a variety of cancers. *See* Col. 4, lines 34-42. (Tab K).

U.S. 6,342,221 teaches an immunoconjugate of an anti-VEGF antibody attached to a therapeutic or diagnostic agent. See claim 14. The therapeutic agent may be a radioactive therapeutic agent. See claim 17. (Tab L).

U.S. Patent No. 5,980,896 teaches immunoconjugates of antibodies and *Pseudomonas* exotoxin A. Claim 22. It also teaches radiolabeled antibodies for imaging cancers. Claims 34-35. (Tab M).

The Nature of the Invention (Factor 4)

From this brief review of the prior art, it is clear that the claimed invention is similar in many technical aspects to techniques which are already used in the art. The art knows how to conjugate antibodies to radiolabels, toxins and drugs. The art knows how to administer such immunoconjugates. The art knows that such immunoconjugates can reach their targets without being degraded or absorbed by non-target tissue. The art knows that sufficient immunoconjugate can reach the targets to have a diagnostic or therapeutic effect. The only new aspect is the association of known protein GPNMB with glioblastomas. Thus, the nature of the invention is that it is a different version of similar technologies.

Consideration of factors 4, 5, 6, and 8 puts the present invention in its proper context within the relevant art. These considerations weigh heavily in favor of a finding of enablement.

The Patent Office has raised a number of issues which are based on speculation alone. Not a single shred of evidence has been adduced to support its theories of why the claimed methods may not work. Yet, such evidence is required to support the rejection. See *In re Marzocchi*, 439 F.2d 2002, 224 (C.C.P.A. 1971). The speculative nature of the issues raised is evident not only from the lack of evidence but from their very grammar:

- "Therapeutic agents may be inactivated *in vivo* before producing a therapeutic effect;"
- "The therapeutic agent may not otherwise reach the target because it may be absorbed;"
- "Circulation into the target area may be insufficient;"
- "A large enough local concentration may not be established."

When the Patent Office fails to meet its burden of raising a genuine issue of lack of operativeness, the burden does not shift to applicants to prove operativeness. *Marzocchi, supra.* Nonetheless, applicants have provided prior art teachings (Tabs B-M) that rebut the naked speculation. Based on the prior art, those of skill in the art would expect that the therapeutic (or diagnostic) agents would not be inactivated before having their effect, that they would reach their targets, that circulation would be sufficient in the target area, and that a sufficient local concentration could be established. Thus, those of skill in the art would have a reasonable expectation of success in practicing the present invention in view of the state of the prior art, the level of skill in the art, the nature of the invention, and the breadth of the claims.

The Office Action asserts that no comparisons between normal cells and cancerous cells of GPNMB expression have been made to demonstrate that it is a cancer specific target. However, Embodiments 2A-2D describe data which demonstrate that GPNMB is glioblastoma

specific. The actual data can be seen in Logging *et al.*, *Genome Research, 10*:133-12000. (Tab N).

SEQUENCE LISTING:

The paper and computer readable forms of the Sequence Listing submitted herewith are identical in content.

Respectfully submitted,

BANNER & WITCOFF, LTD.

Dated: February 9, 2004

By: _____
Sarah A. Kagan
Registration No. 32,141

Banner & Witcoff, Ltd.
Customer No. 22907

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application Of:	)	Group Art Unit: 1642
Gregory J. Riggins *et al.*	)	Examiner: C. Yaen
Serial No.: 09/853,880	)	
Filed: May 14, 2001	)	
For: FOUR GENETIC TUMOR MARKERS SPECIFIC FOR HUMAN GLIOBLASTOMA	)	Atty. Docket No. 000250.000003

DECLARATION OF GREGORY J. RIGGINS

I, Gregory J. Riggins, am an inventor on the above-named application.

I declare that the sequence of SEQ ID NO:17 (Exhibit 1) is the same as the sequence referenced at page 26 of the application as X76534.

X76534 has a "PRI" date of 09-Feb-1995. See Exhibit 2. Thus, this sequence is believed to be the same sequence that was referred to in the specification on the filing date of May 14, 2001.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: February 9, 2004

Gregory J. Riggins

EXHIBIT I

SEQUENCE LISTING

<110> Riggins, Greggory
Lal, Anita
Loging, William

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EXHIBIT II

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VERSION     X76534.1  GI:666042
KEYWORDS    NMB gene.
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            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 2669)
  AUTHORS   Weterman,M.A., Ajubi,N., van Dinter,I.M., Degen,W.G., van
            Muijen,G.N., Ruitter,D.J. and Bloemers,H.P.
  TITLE     nmb, a novel gene, is expressed in low-metastatic human melanoma
            cell lines and xenografts
  JOURNAL   Int. J. Cancer 60 (1), 73-81 (1995)
  MEDLINE   95113576
   PUBMED   7814155
REFERENCE   2
  AUTHORS   Weterman,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-DEC-1993) M. Weterman, University of Nijmegen, Dept
            of Biochemistry, PO Box 9101, 6500 HB Nijmegen, NETHERLANDS
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DESCRIPTION

ZEVALIN™

ZEVALIN™ (Ibritumomab Tiuxetan) is the immunoconjugate resulting from a stable thiourea covalent bond between the monoclonal antibody Ibritumomab and the linker-chelator tiuxetan [N-[2-bis(carboxymethyl) amino]-3-(p-isothiocyanatophenyl)-propyl]- [N-[2-bis(carboxymethyl)amino] -2-(methyl)-ethyl]glycine. This linker-chelator provides a high affinity, conformationally restricted chelation site for Indium-111 or Yttrium-90. The approximate molecular weight of Ibritumomab Tiuxetan is 148 kD.

The antibody moiety of ZEVALIN is Ibritumomab, a murine IgG_1 kappa monoclonal antibody directed against the CD20 antigen, which is found on the surface of normal and malignant B lymphocytes. Ibritumomab is produced in Chinese hamster ovary cells and is composed of two murine gamma 1 heavy chains of 445 amino acids each and two kappa light chains of 213 amino acids each.

ZEVALIN Therapeutic Regimen

The ZEVALIN therapeutic regimen is administered in two steps: Step 1 includes one infusion of Rituximab preceding In-111 ZEVALIN. Step 2 follows Step 1 by seven to nine days and consists of a second infusion of Rituximab followed by Y-90 ZEVALIN.

ZEVALIN™ is supplied as two separate and distinctly labeled kits that contain all of the non-radioactive ingredients necessary to produce a single dose of In-111 ZEVALIN and a single dose of Y-90 ZEVALIN, both essential components of the ZEVALIN therapeutic regimen. Indium-111 chloride and Rituximab must be ordered separately from the ZEVALIN kit. Yttrium-90 Chloride Sterile Solution is supplied by MDS Nordion when the Y-90 ZEVALIN kit is ordered.

http://www.rxlist.com/cgi/generic3/zevalin_ids.htm

ProstaScint Monoclonal Antibody Scan
(111 In-CYT-356)
Utility in the Staging of Prostate Cancer
Introduction

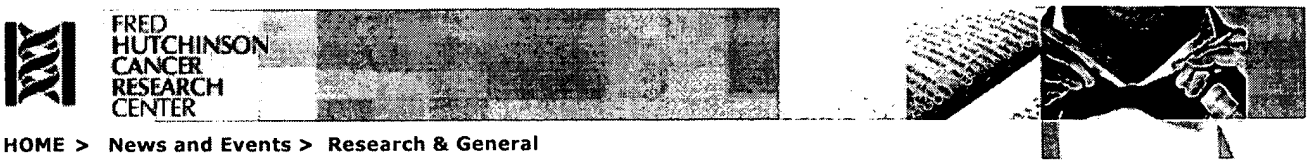
ProstaScint is a monoclonal antibody scanning technique that has implications in the staging of patients newly diagnosed with PC as well as use in evaluating patients believed to have recurrent disease. This monoclonal antibody, or MoAb, reacts with prostate cancer, benign prostatic hypertrophy and to a lesser extent, normal prostate tissue. The MoAb complex is an Indium111 labeled conjugate of the murine MoAb 7E11-C5.3. This antibody appears to recognize a prostate specific membrane glycoprotein that is chiefly expressed by prostatic epithelial cells, both benign and malignant, and whose DNA coding sequence has partial homology to that of the human transferrin receptor. ProstaScint scanning, therefore, involves an intact IgG1 immunoconjugate reactive with prostate specific membrane antigen (PSMA). Patients having a ProstaScint scan are given an iv injection of 0.5 mg of ProstaScint labeled with approximately 5mCi of 111 indium chloride. Initial computerized tomographic images are obtained on the day of the injection and additional tomographic images are acquired on approximately day 4 after injection with many patients undergoing additional scanning between days 5 and 7 to allow time for blood pool and bowel clearance.

http://www.prostate-cancer.org/education/staging/pscint.html

Bexxar

Generic name: Tositumomab and iodine I 131 tositumomab
Manufacturer: Corixa; GlaxoSmithKline
Drug Class: Monoclonal antibody plus radiolabeled monoclonal antibody
Indications: Treatment of patients with CD20 positive, follicular, non-Hodgkin's lymphoma, with and without transformation, whose disease is refractory to rituximab (Rituxan-Genentech) and has relapsed following chemotherapy

http://www.pharmacist.com/new_drug_date/bexxar.cfm



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Contact: Susan Edmonds

206/667-2896

sedmonds@fhcrc.org

Antibody-Targeted Chemotherapy with Mylotarg Shows Promise for Many Adults with Deadly Form of Leukemia

Fred Hutchinson Cancer Research Center Researcher Presents Updated Study Results at the American Society of Clinical Oncology Meeting in New Orleans

NEW ORLEANS, MAY 21, 2000 The first ever antibody-targeted chemotherapy induced remission in a sizable number of adult patients with advanced leukemia, according to data presented by a Fred Hutchinson Cancer Research Center investigator today at the 36th Annual Meeting of the American Society of Clinical Oncology (ASCO). The drug, Mylotarg (gemtuzumab ozogamicin for injection), was recently approved by the U.S. Food and Drug Administration (FDA) to fight relapsed acute myeloid leukemia (AML) for some patients age sixty and older.

AML is a virulent and often fatal form of cancer in which certain white blood cells become cancerous and rapidly accumulate in the bone marrow, preventing normal marrow from growing and functioning properly. It is among the most serious forms of adult leukemia, with a relatively high fatality rate.

"Mylotarg is a new option for many adult patients with AML," said Eric Sievers, M.D., of Fred Hutchinson Cancer Research Center. "Patients greater than 60 years of age achieved remission at a comparable rate to younger patients. If a patient has CD33 positive AML that has relapsed, Mylotarg is worth considering."

Most patients with AML require intensive standard chemotherapy to achieve remission, and some also must undergo bone marrow transplants. Even after such intensive treatment, up to half of all AML patients have residual leukemic cells or experience a relapse. Because standard chemotherapy drugs to treat AML are non-specific - destroying normal as well as malignant cells - patients who receive the therapy tend to become very sick. Researchers at the Fred Hutchinson Cancer

Research Center, and eleven other leading leukemia centers, including University of Chicago Medical Center, MD Anderson Cancer Center, The University of Pennsylvania Cancer Center, Wayne State University, and the City of Hope Medical Center, are working with Wyeth-Ayerst Laboratories and Celltech Chiroscience to study Mylotarg, an antibody-drug conjugate that targets chemotherapy treatment to leukemia cells. In clinical trials, Mylotarg has been referred to as CMA-676.

Mylotarg is the first in a new class of anticancer therapy called antibody-targeted chemotherapy. Mylotargs highly specific antibody recognizes a cell-surface molecule called "CD33" which is abundant on AML cells but absent from normal blood stem cells, the seeds from which normal blood and immune cells originate. This engineered antibody is linked to a novel and extremely potent chemotherapy agent known as "calicheamicin". The antibody selectively targets leukemic blast cells and delivers calicheamicin to them. As a result, the leukemic cells are destroyed but the cells that are responsible for replenishing normal blood cells are spared.

Pooled data from three Phase II clinical trials reported on 104 patients, each of whom had CD33 positive AML in first relapse. Mylotarg produced remission in 31 percent of patients - a rate comparable to that of standard combination chemotherapy regimens - with mild and tolerable side effects. When evaluating the patients with respect to age, remission was achieved in 34 percent of patients under 60, and 28 percent of patients sixty and older. There were no clinically significant differences in side effects between the age groups.

"Most striking was the near absence of severe mucositis, or mouth sores seen in association with Mylotarg treatment," Sievers continued. "While standard chemotherapy induction is usually associated with a prolonged hospital admission, one-sixth of the patients who received Mylotarg were hospitalized for less than a week."

Standard combination chemotherapy treatment often produces significant major organ damage, and sores both in the mouth and in the intestinal tract (frequent sources for opportunistic infections), but Mylotarg treatment does not. Mylotarg also is associated with a relatively low treatment-related mortality. As with standard chemotherapy treatments, Mylotarg produces a temporary suppression of bone marrow and blood cell counts.

Mylotarg is administered in two IV infusions fourteen days apart, and many patients have received the drug on an outpatient basis. Unlike standard chemotherapy regimens, which involve multiple drugs, Mylotarg was given alone.

The Fred Hutchinson Cancer Research Center is an independent, non-profit research institution dedicated to the development and advancement of biomedical technology to eliminate cancer and other

potentially fatal diseases. Recognized internationally for its pioneering work in bone marrow transplantation, the Center has four scientific divisions collaborating to form a unique environment for conducting basic and applied science. One of 35 National Cancer Institute-designated comprehensive cancer centers in the country, it is the only one in the Northwest. Visit the Hutchinson Center web site for more information at www.fhcrc.org.

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Advancing knowledge, saving lives

Editor's Note: Backgrounders are available on the CD33 monoclonal antibody and calicheamicin.

For information about Wyeth-Ayerst Laboratories, contact: Douglas Petkus, 610/971-4980.

Mylotarg: Mylotarg is a trademark of American Home Products Corp.

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Advancing knowledge, saving lives

The Fred Hutchinson Cancer Research Center is an independent, nonprofit research institution dedicated to the development and advancement of biomedical technology to eliminate cancer and other potentially fatal diseases. Recognized internationally for its pioneering work in bone-marrow transplantation, the Center's four scientific divisions collaborate to form a unique environment for conducting basic and applied science. The Hutchinson Center is the only National Cancer Institute-designated comprehensive cancer center in the Pacific Northwest. For more information, visit the Center's Web site at <www.fhcrc.org>.

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Internet Services
(206) 667-7999
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FRED HUTCHINSON CANCER RESEARCH CENTER
1100 Fairview Ave N P.O. Box 19024
Seattle Washington 98109
206.667.5000

1: Curr Opin Pharmacol. 2003 Aug; 3(4): 386-90. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Antibody-targeted chemotherapy with immunoconjugates of calicheamicin.

Damle NK, Frost P.

Oncology Discovery Research, Wyeth Research, 401 North Middletown Road, Pearl River, NY 10965, USA. damlen@wyeth.com

Targeted delivery of cytotoxic agents to tumours is believed to improve both their anti-tumour efficacy and their safety. Antibodies specific for tumour-associated antigens have been used to deliver cytotoxic agents to tumour cells. Calicheamicin is a potent cytotoxic agent that causes double-strand DNA breaks, resulting in cell death. When conjugated to monoclonal antibodies specific for tumour-associated antigens, calicheamicin exerts strong antigen-specific anti-tumour effects against human tumour xenografts in preclinical models. Antibody-targeted chemotherapy with immunoconjugates of calicheamicin, exemplified by gemtuzumab ozogamicin (Mylotarg), is a clinically validated therapeutic strategy for the treatment of human cancer.

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- Review
- Review, Tutorial

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Medical Sciences

Recombinant immunotoxins specific for a mutant epidermal growth factor receptor: Targeting with a single chain antibody variable domain isolated by phage display

(EGFRvIII/glioblastoma/cancer therapy/Pseudomonas exotoxin A)

IAN A. J. LORIMER*, ANDREA KEPPLER-HAFKEMEYER*, RICHARD A. BEERS*, CHARLES N. PEGRAM†, DARELL D. BIGNER†, AND IRA PASTAN*‡

*Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, 37 Convent Drive, MSC 4255, Bethesda, MD 20892-4255; and †Department of Pathology, Duke University Medical Center, 177 Medical Sciences Research Building, Research Drive, Durham, NC 27710

Contributed by Ira Pastan, September 23, 1996

ABSTRACT **EGFRvIII is a mutant epidermal growth factor receptor found in glioblastoma, and in carcinoma of the breast, ovary, and lung. The mutant receptor has a deletion in its extracellular domain that results in the formation of a new, tumor-specific extracellular sequence. Mice were immunized with a synthetic peptide corresponding to this sequence and purified EGFRvIII. A single chain antibody variable domain (scFv) phage display library of 8×10^6 members was made from the spleen of one immunized mouse. A scFv specific for EGFRvIII was isolated from this library by panning with successively decreasing amounts of synthetic peptide. This was used to make an immunotoxin by fusing the scFv DNA sequence to sequences coding for domains II and III of Pseudomonas exotoxin A. Purified immunotoxin had a K_d of 22 nM for peptide and a K_d of 11 nM for cell-surface EGFRvIII. The immunotoxin was very cytotoxic to cells expressing EGFRvIII, with an IC_{50} of 1 ng/ml (16 pM) on mouse fibroblasts transfected with EGFRvIII and an IC_{50} of 7–10 ng/ml (110–160 pM) on transfected glioblastoma cells. There was no cytotoxic activity at 1000 ng/ml on the untransfected parent glioblastoma cell line. The immunotoxin was completely stable upon incubation at 37°C for 24 h in human serum. The combination of good affinity, cytotoxicity and stability make this immunotoxin a candidate for further preclinical evaluation.**

Immunotoxins are therapeutic agents for cancer that consist of a targeting molecule linked to a cytotoxic agent (1). Antibodies, or genetically engineered antibody variable domains (Fvs), are usually used for targeting. Our laboratory has focused on using the protein Pseudomonas exotoxin A as a cytotoxic agent. Pseudomonas exotoxin A is a three-domain protein (2): domain I binds to the $\alpha 2$ macroglobulin receptor, which internalizes the toxin (3); domain II mediates translocation of the toxin to the cell cytosol; domain III ADP ribosylates elongation factor 2 leading to arrest of protein synthesis and cell death. The toxin can be converted to a cancer therapeutic by replacing domain I with binding domains that are selective for cancer cells. Recently our laboratory completed a phase I trial of an immunotoxin made with an antibody attached to domains II and III of Pseudomonas exotoxin A (4). The antibody was specific for a Lewis Y-related carbohydrate antigen that is overexpressed in many cancers. Tumor regressions were seen in several patients with advanced breast and colorectal cancer, demonstrating that immunotoxins made with Pseudomonas exotoxin A do have activity against solid tumors in humans.

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The immunotoxin used in this phase I trial was made using a whole mouse monoclonal antibody chemically coupled to toxin. Disadvantages of this type of immunotoxin are the large size (≈200 kDa) which can result in poor tumor penetration (5), the heterogeneity of the linkage between antibody and toxin, and the difficulty of producing the conjugates in large amounts. To overcome these problems we have made wholly recombinant immunotoxins in which cloned Fvs are used for targeting (6, 7). Previously this has been done by cloning variable domains from specific hybridomas and converting them to single chain Fvs (scFvs) (8, 9) or disulfide-linked Fvs (10) by genetic engineering methods. Problems with this approach were that many antibody variable domains functioned poorly as scFvs, either because of instability due to a weak V_H–V_L association, or poor binding due to problems such as interference by the linker sequence. Here we have used phage display technology to bypass the hybridoma step and isolate scFvs directly. In phage display, peptides or proteins are expressed on the surface of phage as fusion proteins (11). This allows the selection and amplification of phage clones with specific binding activities.

Immunotoxins have been made that recognize a wide variety of cell-surface targets on cancer cells. Typically these are tumor-associated antigens—i.e., antigens that are overexpressed on cancer cells relative to normal cells. This study concerns the development of immunotoxins that target a mutant epidermal growth factor receptor (EGFR) which is only expressed on tumor cells. This mutant receptor was first detected in human glioblastoma cells. Early work on the genetic changes that take place in these cells showed that the EGFR gene is often amplified in these cells (12). Further studies showed that the gene is also frequently rearranged (13–15). The most common rearrangement was a deletion of exons 2–7. This deletion is in-frame, and a new glycine codon is formed at the deletion junction. The mutant receptor, designated EGFRvIII, is expressed on the cell surface and contains a new tumor-specific protein sequence in its extracellular domain. It has also been shown to have constitutive protein kinase activity (16). EGFRvIII cDNA has the properties of an oncogene; it transforms 3T3 mouse fibroblasts (15) and enhances the *in vivo* malignancy of human glioblastoma cells (16). EGFRvIII has been found in 50–60% of glioblastomas (17, 18). More recently it has also been shown to be present in 70–80% of breast and ovarian cancers (17, 18), and 16% of non-small cell lung cancers (19). The frequent expression of EGFRvIII in human tumors make it an attractive target

Abbreviations: IC_{50}, 50% inhibitory concentration; Fv, antibody variable domain; scFv, single chain Fv; EGFR, epidermal growth factor receptor.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U76382).

‡To whom reprint requests should be addressed.

for therapeutics. A peptide corresponding to the tumor-specific extracellular sequence is immunogenic in animals, and both polyclonal (13) and monoclonal antibodies (17, 20) have been raised that are specific for this sequence and for the mutant receptor.

Previously we have shown that EGFRvIII-specific antibodies chemically coupled to domains II and III of Pseudomonas exotoxin A were cytotoxic to cells expressing EGFRvIII, but had little or no activity against cells expressing wild-type EGFR (21). We next attempted to make wholly recombinant toxins that used only antibody Fv domains for targeting (I.A.J.L., C.N.P., D.D.B., and I.P., unpublished data). The variable regions of two EGFRvIII-specific antibodies were cloned and used to make immunotoxins. These were active, but in both cases had reduced binding compared with the original antibody, and reduced cytotoxicity compared with the whole antibody immunotoxins. In this study, we have used phage display to isolate a high-affinity EGFRvIII-specific Fv domain directly from an immunized mouse. This Fv was used to make an immunotoxin that had greatly improved cytotoxicity compared with the recombinant immunotoxins made previously.

MATERIALS AND METHODS

Mouse Immunization. Ajax mice were immunized s.c. with 30 μg of peptide-3/keyhole limpet hemocyanin conjugate in complete Freund's adjuvant. Peptide 3 has as its sequence the first 13 amino acids of the EGFRvIII N terminus plus a carboxyl-terminal cysteine (17). After 80 days, mice were boosted with 30 μg of peptide-3/keyhole limpet hemocyanin conjugate in incomplete Freund's adjuvant containing 1 mg of Salmonella Minnesota (Ribi Immunochem) per ml. Mice were given three further s.c. boosts at ≈80-day intervals with 20 μg of peptide-3/keyhole limpet hemocyanin conjugate and 20 μg of affinity-purified EGFRvIII in incomplete Freund's adjuvant. After 5.5 months, mice were given a boost i.p. with 20 μg of peptide-3/keyhole limpet hemocyanin conjugate and 30 μg of affinity-purified EGFRvIII without adjuvant. This was repeated 2 months later. Spleens were harvested 5 days after the final boost.

Library Construction. Splenocytes were purified on a Ficoll cushion and poly(A)$^+$ RNA was purified using a FastTrack mRNA isolation kit from Invitrogen. A scFv library was constructed in the vector pCANTAB5E using the recombinant phage antibody system from Pharmacia following the manufacturer's protocol. One-third of the final ligation reaction was used to transform TG1 cells by electroporation.

Synthetic Peptide. The synthetic peptide LEEKKGNYVVTDHSGGK-biotin was synthesized by Peptide Technologies (Gaithersburg, MD). The first 13 amino acids correspond to the N terminus of EGFRvIII and contain the tumor-specific deletion junction sequence. The SGG sequence is intended to function as a flexible spacer. The carboxyl-terminal residue is a lysine with biotin attached to the ε-amino group.

Panning Procedure. *Escherichia coli* TG1 (Stratagene) were electroporated and grown in 10 ml 2× YT medium (16 g bacto-tryptone/10 g bacto-yeast extract/5 g NaCl per liter in H_2O) plus 2% glucose with shaking for 1 h at 37°C. Ampicillin (final concentration, 100 μg/ml) and M13KO7 helper phage (4×10^{10} plaque-forming units) were then added, and the culture was grown for one more hour. Cells were then pelleted and resuspended in 10 ml 2× YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. The culture was then incubated overnight with shaking at 37°C. Phage were purified from the culture supernatant by precipitation three times with polyethylene glycol, resuspended in PBS, titered, and stored at −70°C until panning. The panning procedure used is a modification of the method described by Schier *et al.* (22). Phage (5×10^{11}) in 0.5 ml PBS were mixed with 0.5 ml of 4% skim milk powder/0.05% Tween 20/PBS containing biotinylated peptide. This was mixed end-over-end at room temperature for 1 h and then added to 300 μl of Dynabeads M-280 streptavidin-coated magnetic beads (Dynal, Great Neck, NY) that had been blocked for 1 h at 37°C with 2% skim milk powder/0.025% Tween 20/PBS. After a 15-min incubation at room temperature, magnetic beads were captured with a magnet and washed six times with 0.05% Tween 20 in PBS and four times with PBS. Beads were resuspended in 600 μl PBS and half of this was used directly to reinfect log phase TG1 cells.

Phage and scFv ELISA. Immulon 4 plates were coated overnight at 4°C with 200 μl per well 10 μg/ml streptavidin (Pierce). Plates were then washed three times with 0.05% Tween 20 in PBS. A total of 200 μl per well of 1 μM EGFRvIII peptide was added for 15 min at room temperature, and the plate was washed again. A total of 200 μl per well of blocking buffer (2% milk powder in PBS) was added for 1 h at room temperature. Phage or periplasm (see below) in blocking buffer was added for 1 h at room temperature. The plate was then washed three times as above. For phage ELISA, 200 μl per well of antiM13 antibody-horse radish peroxidase conjugate (Pharmacia) in blocking buffer, diluted according to the manufacturer's instructions, was added; for periplasm, 200 μl per well of anti-Etag antibody-horse radish peroxidase conjugate (Pharmacia) in blocking buffer was added (1:8000 dilution). After 1 h at room temperature, plates were washed, and 200 μl per well of 2,2′-azinobis-[3-ethylbenzothizoline-6-sulfonic acid] (ABTS) substrate solution was added. After 15 min, absorbance in wells was determined by using an ELISA plate reader.

Periplasm Preparation. *E. coli* HB2151 was infected with ELISA-positive phage. 5 ml of an overnight culture (from a single colony) was used to start a 1 l culture in 2× YT medium containing 2% glucose and 100 μg/ml ampicillin. This was grown to an A_{600} of 0.9, at which point cells were pelleted and resuspended in 1 liter of 2× YT medium containing 1 mM isopropyl β-D-thiogalactoside and 100 μg/ml ampicillin. After 3 h of growth at 37°C, cells were pelleted and resuspended in 20 ml of ice-cold TES buffer (0.2 M Tris·HCl/0.5 mM EDTA/0.5 M sucrose, pH 8.0). Thirty-five milliliters of cold water was added and cells were incubated on ice for 30 min. Cells were then removed by centrifugation, and the supernatant was recentrifuged at 14,000 rpm in a Sorvall SS34 rotor for 30 min. The supernatant was dialyzed overnight against 2 liters of PBS and stored at −70°C.

Sequencing. Sequencing was done as described (23) by using an Applied Biosystems model 373A DNA sequencing system.

scFv Immunotoxin Plasmid Construction, Expression, and Purification. A scFv immunotoxin was made as described by fusing the scFv to domains II and III of Pseudomonas exotoxin A (7). The scFv immunotoxin was expressed under control of the T7 promoter in *E. coli* BL21(λ DE3). Purification of inclusion bodies, refolding, and immunotoxin purification by ion-exchange and size-exclusion chromatography were all as described (7).

Surface Plasmon Resonance. Binding to EGFRvIII peptide was measured by surface plasmon resonance (24) using the Pharmacia BIAcore 1000 with UPGRADE system. A total of 1800–2400 resonance units of streptavidin (Pierce) were coupled to the surface of carboxylated dextran-coated CMS research grade chips at pH 4.8 using the amide coupling reagents provided with the BIAcore system. Biotinylated EGFRvIII peptide was then bound to the streptavidin by flowing 10 or 100 nM solutions of peptide over the chip. Streptavidin binding sites were not saturated with the lower concentration of peptide. Solutions of MR1 immunotoxin (12.5, 25, or 50 μg/ml) were then passed over the chip to measure binding. Regeneration was done with 30 mM phosphoric acid. Nonspecific binding was determined using a

streptavidin chip without peptide. Binding kinetics were analyzed by using BIAcore 1000 with UPGRADE software.

Cell Binding Assays. MR1 immunotoxin (6 μg) was labeled with Bolton–Hunter reagent and purified on a PD10 column (Pharmacia) equilibrated with PBS containing 0.5% gelatin. The specific activity was 9×10^5 cpm/μg. Binding assays were performed by incubating NR6M cells at 4°C with varying amounts of labeled immunotoxin in DMEM containing 20 mM Hepes (pH 7.4) and 0.2% gelatin. After 2 h, cells were washed four times with 0.2% BSA in PBS to remove unbound immunotoxin. Bound immunotoxin was solubilized by incubation for 30 min at room temperature with 2% Triton X-100/0.2% BSA in PBS and counted in a γ counter. Nonspecific binding was determined by measuring binding in the presence of 10 μM EGFRvIII peptide. The binding dissociation constant K_d was determined by Scatchard analysis, using a least squares fit to a straight line.

Cell Culture and Cytotoxicity Assays. The culture of NR6M cells was as described (21). U87MG and U87MG.ΔEGFR human glioblastoma cells were from W. Cavenee (University of California at San Diego) (16). They were grown in RPMI 1640 medium containing 10% fetal bovine serum (supplemented with 375 μg/ml G418 for the transfectant). Cytotoxicity assays were done as described (21), except that glioblastoma cells were labeled for 6 h with [^{3}H]leucine.

RESULTS

Immunization, Library Construction, and Panning. Mice were immunized with EGFRvIII peptide coupled to keyhole limpet hemocyanin and boosted with peptide and purified EGFRvIII receptor as described. Ajax mice were used because they showed a better response to peptide immunization than other mouse strains. Immunized mice showed high titers of antibody against EGFRvIII when tested by ELISA against EGFRvIII peptide. A scFv phage library was made from the spleen of one mouse, which had an endpoint serum titer in ELISA against EGFRvIII peptide of more than 1/100,000 14 days after the next to last antigen boost. A test transformation of the final ligation reaction showed that a library of 8×10^6 members could be made. One third of this ligation reaction (2.4×10^6 members) was used to transform *E. coli* for panning. The panning system used was chosen to select for a scFv with a high affinity for EGFRvIII. Panning was done in solution to avoid problems with selection on the basis of avidity that have been reported to occur with scFv libraries (22). Two rounds of panning were done with a high concentration of peptide (1 μM) to select for EGFRvIII binding phage. The number of phage captured on the magnetic beads increased 400-fold with the second round of panning (see Table 1). We then panned with successively decreasing concentrations of peptide (1 nM, 0.1 nM, and 0.01 nM) to select for phage with the highest affinity for EGFRvIII. The number of phage captured with 0.01 nM peptide was very low (Table 1), so we chose to analyze the phage captured from the previous round done with 0.1 nM peptide.

Characterization of Phage Clones. Single colonies from the titration of captured phage were used to rescue 20 individual phage clones. These were analyzed by phage ELISA. None showed binding to streptavidin in the absence of EGFRvIII peptide. Nineteen of 20 showed binding in the presence of peptide. Ten ELISA-positive phage were used to prepare phagemids. The PCR was used to make scFv DNA from each of these. All of these had the fragments of the same size after digestion with *Bst*NI restriction enzyme (not shown), suggesting that the clones were the same or very similar. Three of these were sequenced and found to be identical. This sequence has been deposited in the GenBank data base. The scFv coded for by this sequence was designated MR1 (for mutant receptor). These results show that, after four rounds of panning, we had selected for a phage population that consisted predominately of one clone. A single clone was used to make a crude periplasmic preparation of scFv protein with a C-terminal epitope tag (see *Materials and Methods*). This also showed specific binding to EGFRvIII peptide in an ELISA assay.

Table 1. Panning of scFv library with EGFRvIII peptide

Round	Concentration peptide, nM	No. of phage panned	No. of phage eluted
1	1000	5×10^{11}	9×10^4
2	1000	5×10^{11}	3.7×10^7
3	1	5×10^{11}	2.1×10^6
4	0.1	5×10^{11}	9×10^5
5	0.01	2.5×10^{11}	2.4×10^4

ScFv Immunotoxin Expression and Purification. The scFv sequence was used to construct a plasmid for expression of a scFv immunotoxin (7). In this, the scFv sequence is fused to DNA for domains II and III of Pseudomonas exotoxin A. The version of Pseudomonas exotoxin A used here, PE38KDEL, has a modified C terminus which enhances its cytotoxicity (25). With this plasmid, expression is driven by the T7 promoter. MR1 immunotoxin was expressed as inclusion bodies in *E. coli* and could be detected as the major band on SDS/PAGE of solubilized whole cell. Inclusion bodies were purified, solubilized in 6 M guanidine HCl, and refolded (26). Refolded protein was purified by Q-Sepharose and MonoQ ion-exchange chromatography, followed by size exclusion chromatography. The yield of refolded immunotoxin was ≈2% (expressed as mg protein after MonoQ chomatography/mg inclusion body protein). The immunotoxin migrated as a monomer on size exclusion chromatography and migrated as a single band of the expected size (M_r 63,599) on SDS/PAGE (Fig. 1).

Binding. Binding of MR1 immunotoxin to synthetic peptide was measured by surface plasmon resonance (24). Streptavidin was first coupled to the chip, and the biotinylated peptide was then bound to this. This chip could be regenerated repeatedly because of the very high affinity of biotin-streptavidin binding. Values for on rates and off rates were determined for three different concentrations of immunotoxin and two different concentrations of peptide on the chip. Calculated K_d values were consistent under the different conditions used. k_{on} was 1.28×10^5 ($\pm 0.15 \times 10^5$) $M^{-1} \cdot sec^{-1}$. k_{off} was 2.77×10^{-3} ($\pm 0.18 \times 10^{-3}$) sec^{-1}. The K_d calculated from these is 22 (±4) nM. (Each value is the mean of six determinations ± SD.)

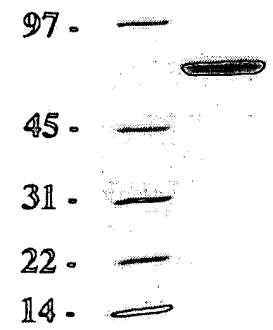

FIG. 1. SDS/PAGE of purified MR1 immunotoxin. MR1 immunotoxin (5 μg) was electrophoresed on a 4–20% gradient gel. Migration of molecular weight markers (in kDa) is shown on the left.

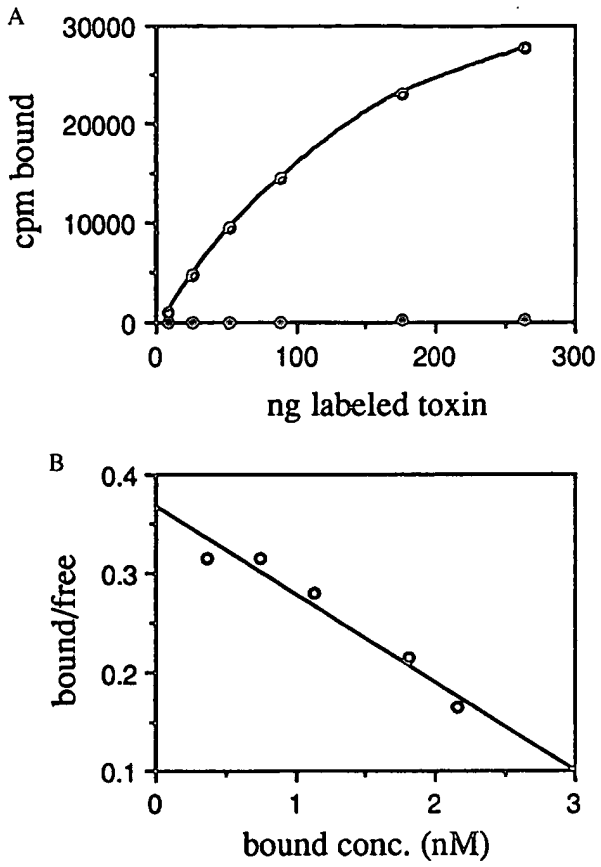

FIG. 2. Binding of MR1 immunotoxin to NR6M cells. MR1 immunotoxin was labeled with ^{125}I and binding to live cells at 4°C was assayed. (*A*) Binding in the absence (○) or presence (●) of 10 μM EGFRvIII peptide. (*B*) The same data replotted for Scatchard analysis. The slope was 0.088 nM^{-1}, giving a K_d of 11 nM; the regression coefficient was 0.947.

We also measured binding of iodinated MR1 immunotoxin to the cell line NR6M (Fig. 2*A*). This is a Swiss 3T3 cell line selected for lack of expression of mouse EGFR and transfected with EGFRvIII cDNA (21). The labeled immunotoxin bound to NR6M cells, and binding could be competed by EGFRvIII peptide, showing that it was specific. Scatchard analysis of this data (Fig. 2*B*) gave a K_d of 11 nM. Number of sites per cell was 3×10^5 in agreement with previously published values (21).

Cytotoxicity. The cytotoxicity of MR1 immunotoxin was first assayed using NR6M cells. To assess cytotoxicity, we have used inhibition of protein synthesis by Pseudomonas exotoxin as a surrogate measure. We have found previously that this corresponds well with cytotoxicity. MR1 immunotoxin inhibited 50% of protein synthesis at a concentration of 1 ng/ml (16 pM) when cells were exposed to immunotoxin for 24 h (Fig. 3*A* and Table 2). The IC_{50} was 30 ng/ml after cells were exposed for 2 h to immunotoxin, and 0.5–0.6 ng/ml after a 48-h exposure. This shows that the immunotoxin remains active in tissue culture for greater than 24 h. The stability of the immunotoxin in human serum was also evaluated; MR1 immunotoxin was incubated at 20 μg/ml in human serum at 37°C for various periods of time and then assayed for activity (Fig. 3*B*). There was no loss of activity after 24 h, showing that the scFv is very stable. The cytotoxicity of MR1 immunotoxin could be inhibited by 5 μM EGFRvIII peptide, showing that it was specific (Fig. 3*C*).

The cytotoxicity of MR1 immunotoxin was also tested on U87MG cells transfected with EGFRvIII (16). U87MG is a human glioblastoma line and represents a more relevant cell line to assess the therapeutic potential of MR1 immunotoxin. The transfectants express 2×10^5 normal EGFRs and 4×10^5 EGFRvIII per cell by FACS analysis (C. Wikstrand and D.D.B., unpublished observations). The IC_{50} of MR1 immunotoxin on these cells was 7–10 ng/ml (110–160 pM) (Fig. 3*D* and Table 2). No cytotoxicity was seen on the untransfected parent cell line at concentrations up to 1000 ng/ml. This clearly demonstrates the specificity of MR1 immunotoxin for the mutant EGFR.

We also made a version of MR1 immunotoxin that had the naturally occurring carboxyl terminus of Pseudomonas exotoxin A, REDLK, instead of KDEL. This was used to assess the influence of the KDEL modification on the activity of MR1 immunotoxin. MR1 immunotoxin with REDLK carboxyl terminus was 3- to 4-fold less active on both NR6M cells and U87MG transfectants (Table 2).

DISCUSSION

The mutant EGFR EGFRvIII is a promising target for cancer therapy, because it has a tumor-specific extracellular sequence and it occurs frequently in a number of different tumor types. Here we have used phage display to directly isolate an EGFRvIII-specific scFv from an immunized mouse spleen. The immunotoxin made with this scFv had a higher binding affinity than the recombinant immunotoxins made previously. The K_d was 22 nM for the peptide and 11 nM for the cell surface receptor. The numbers are very similar, suggesting that scFv interacts with the peptide and receptor N-terminal sequence in the same way. The two values were determined by very different techniques; therefore we cannot tell if the small difference in affinity is due to differences in methodologies, or represents a true difference in affinity of the scFv for the receptor. (We have considered the latter possibility because boosts during immunization were done with both peptide and purified EGFRvIII.) ScFvs with K_d values of as low as 0.3 nM have been reported in the literature (27); it may therefore be possible to make even better EGFRvIII-specific immunotoxins, either by isolating new scFvs with higher affinities, or by improving the affinity of MR1.

MR1 immunotoxin also had better cytotoxic activity than the recombinant immunotoxins made previously, with an IC_{50} of 16 pM on transfected mouse fibroblasts. MR1 immunotoxin was 7- to 10-fold less active on the human glioblastoma cell transfectants. Since the two cell lines express similar levels of EGFRvIII, a difference in receptor numbers cannot explain this result. One difference between the two cell lines is that the glioblastoma cells express both wild-type and mutant receptor. We considered the possibility that the wild-type receptor interacts with the mutant receptor and decreases its rate of internalization. We found that addition of EGF during the assay, which would promote internalization of wild-type EGFRs, did not enhance the cytotoxicty of immunotoxins targeting EGFRvIII. Also U87MG cells tended to be more resistant to killing by a number of other immunotoxins targeting ubiquitously expressed receptors. On this basis we feel that the lower sensitivity of the glioblastoma cells is due to events downstream of receptor binding and internalization. This would include steps such as proteolytic processing, retrograde transport to the endoplasmic reticulum, and transport to the cytosol. However MR1 immunotoxin is still quite cytotoxic to the glioblastoma transfectants, and shows no cytotoxicity to glioblastoma cells that do not express EGFRvIII.

MR1 immunotoxin was also very stable. Cytotoxicity in tissue culture was proportional to the length of time the cells were exposed to immunotoxin over at least a 48-h period. There was no loss of activity after incubation at 37°C for 24 h in human serum. This contrasts with our findings with another

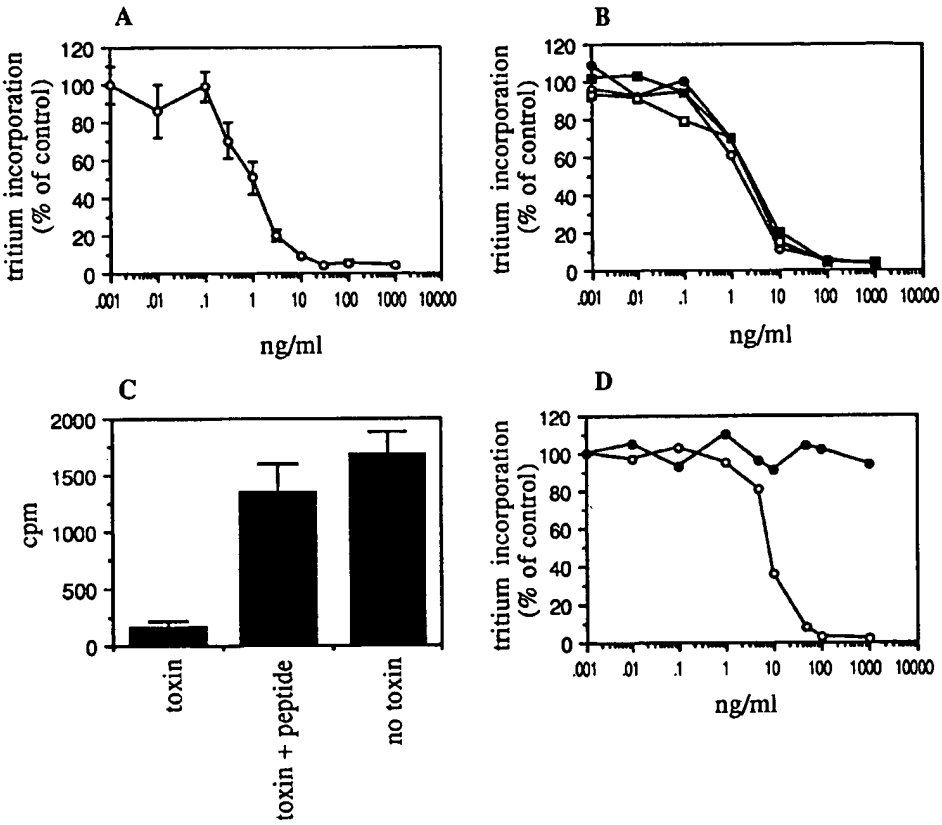

FIG. 3. (*A*) Cytotoxicity of MR1 immunotoxin to NR6M cells. Cells were exposed for 24 h to immunotoxin, and incorporation of [^{3}H]leucine was measured. (*B*) Stability of MR1 immunotoxin. Immunotoxin was incubated at 20 μg/ml in human serum for 0 h (○), 4 h (●), 8 h (□), or 24 h (■) at 37°C. Cytotoxic activity was then measured as in *A*. (*C*) Inhibition by peptide. Cytotoxicity assays were performed with 10 ng/ml MR1 immunotoxin in the presence or absence of 5 μM EGFRvIII peptide. (*D*) Cytotoxicity of MR1 immunotoxin to human glioblastoma cells transfected with EGFRvIII. ○, U87MG.ΔEGFR transfectant; ●, U87MG untransfected parent cell line. Each data point shown is the mean of triplicate determinations; error bars show SEM.

scFv immunotoxin, which lost almost all of its activity after 24 h when assayed under the same conditions (10). We have found that many scFvs made from monoclonal antibodies are unstable, and that this can limit their antitumor activity in animal models (10). Differences in the stability of scFvs are mainly due to variations in the strength of the V_H–V_L interface in different antibodies. It seems likely that phage display selects directly for stable scFv. Standard phage rescue procedures involve overnight incubations at 37°C, which probably inactivate many unstable scFv. The process of secretion of scFv gene III fusion proteins across the bacterial inner membrane during phage assembly may also favor more stable scFvs. The stability of MR1 may be related to its 11-amino acid heavy chain CDR3, which is relatively long compared with the average length of 9 amino acids for mice (28). It has been shown that there is a correlation (albeit a weak one) between HCDR3 length and the surface area of the V_H–V_L interface (29).

Table 2. Cytotoxicity of immunotoxins on different cell lines

Immunotoxin	Cell lines		
	NR6M	U87MG.ΔEGFR	U87MG
MR1scFvPE38KDEL	1 (16)	7–10 (110-160)	>1000
2 h	30 (480)		
48 h	0.5–0.6 (8-10)		
MR1scFvPE38REDLK	3–4 (48-64)	40 (640)	>1000

The concentration of immunotoxin at which 50% inhibition of protein synthesis occurs (IC_{50}) is given in ng/ml with the value in pM in parentheses. All values are for 24-h exposure to immunotoxin unless stated otherwise.

In summary, MR1 immunotoxin is stable and is cytotoxic to cells expressing EGFRvIII, including human glioblastoma cells. We have previously shown that another Pseudomonas exotoxin A-based immunotoxin, LMB7, is active in a nude rat model of carcinomatous meningitis when administered intrathecally (30). MR1 immunotoxin appears to be a good candidate for testing as a therapeutic agent for primary brain cancer using a similar animal model. MR1 scFv may also be useful for targeting of radioisotopes or other therapeutic agents to EGFRvIII-expressing tumors.

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Medical Sciences

Intrathecal administration of single-chain immunotoxin, LMB-7 [B3(Fv)-PE38], produces cures of carcinomatous meningitis in a rat model

IRA H. PASTAN*, GARY E. ARCHER†, ROGER E. MCLENDON†, HENRY S. FRIEDMAN†‡, HERBERT E. FUCHS†, QING-CHENG WANG*, LEE H. PAI*, JAMES HERNDON§, AND DARELL D. BIGNER†‡

*Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, National Institutes of Health, 37 Convent Drive, MSC 4255, Bethesda, MD 20892-4255; and †Department of Pathology, ‡The Preuss Laboratory for Brain Tumor Research, and §Community and Family Medicine, Division of Biometry, Duke University Medical Center, Durham, NC 27710

Contributed by Ira H. Pastan, December 22, 1994

ABSTRACT **LMB-7 [B3(Fv)-PE38] is a single-chain immunotoxin constructed from the murine monoclonal antibody B3 and a truncated form of *Pseudomonas* exotoxin PE38. Antibody B3 recognizes a carbohydrate epitope found on solid tumors that frequently invade the intrathecal space and cause neoplastic meningitis. We tested the therapeutic value of intrathecally administered LMB-7 by using a model of human neoplastic meningitis in athymic rats. This model is representative of a clinical situation in that antibody B3 cross-reacts with a number of normal tissues that can be used to monitor potential systemic toxicity. Treatment was begun 3 days after A431 tumor implantation. Without treatment, the animals median survival was 10 days. Intrathecal administration of 10 μg of LMB-7 in 40 μl on days 3, 5, and 7 produced 4 of 10 and 8 of 10 long-term survivors (>170 days) in two experiments. Of the long-term survivors, 2 of 4 and 7 of 8 survivors had no microscopic evidence of tumor and were considered histologic cures. Lack of significant toxicity in the effective dose range and specificity make LMB-7 an excellent candidate for intrathecal treatment of neoplastic meningitis in humans.**

At one time neoplastic meningitis was thought to be a rare complication, but with improvements in systemic cancer treatment and an increased awareness of intrathecal complications, an increase in the number of cases of neoplastic meningitis has been seen. The intrathecal compartment provides a reservoir for tumor growth, most likely due to a failure of systematically administered chemotherapeutic agents to reach a therapeutic level in the cerebrospinal fluid (1). To overcome systemic delivery limitations, direct infusion into the intrathecal space has been used.

Monoclonal antibodies (mAbs) conjugated to specific radionuclides, drugs, and toxins are being actively investigated as therapeutic agents in the compartmental treatment of neoplastic meningitis. Immunotoxins are particularly appealing because they are not affected by tumor cell hypoxia as are some radiolabeled mAbs, and they are more efficient than mAb–drug conjugates (2). We have observed significant increases in survival by using a model of human neoplastic meningitis in athymic rats, in animals treated with LMB-1, an immunotoxin constructed with the intact IgG of mAb B3 and a truncated form of *Pseudomonas* exotoxin (D.D.B., G.E.A., R.E.M., H.S.F., H.E.F., L.H.P., J.H., and I.H.P., unpublished data). mAb B3 was chosen for this study because it reacts with many types of solid tumors including carcinomas of the colon, breast, lung, ovary, bladder, and stomach (3). Recombinant techniques have made it possible to isolate the antigen-binding variable regions of the light and heavy chains from a mAb and connect them with a flexible linker to form a single-chain antigen binding protein, termed sFv.

For this study, we have used a recombinant immunotoxin in which the sFv of mAb B3 is fused to PE38, an altered form of *Pseudomonas* exotoxin (4), to give LMB-7 or [B3(Fv)-PE38]. Previous studies have shown (5) that LMB-7 administered i.v. produced complete regression of large subcutaneous tumors arising from A431 epidermoid carcinoma cells and MCF-7 breast carcinoma cells. By using a human neoplastic meningitis model, we have now tested the therapeutic efficacy of this single-chain immunotoxin LMB-7. The nude rat is an excellent experimental model for this purpose because human cancer cells grow and produce meningitis and because some normal rat tissues such as stomach, lung, and pars intermedia of the pituitary express the Ley antigen, partially mimicking normal antigen distribution in humans. We report here that LMB-7 given intrathecally 3, 5, and 7 days after tumor implantation increases median survival from 10 days to >190 days, at which point the experiment was terminated.

MATERIALS AND METHODS

Animal Model. Subarachnoid catheters were implanted in athymic rats by using the method of Fuchs *et al.* (6). Neoplastic meningitis was initiated by injection of 5×10^6 tumor cells in phosphate-buffered saline through an indwelling subarachnoid catheter. The target cell line for these experiments is the human epidermoid carcinoma line A431, which homogenously expresses antigens with which mAb B3 reacts.

Treatment Studies. LMB-7 was prepared as described (7) at a final concentration of 0.25 mg/ml. In the initial activity studies, groups of 10 animals were treated with a dose (in 40 μl) of 10 μg of LMB-7 or an equal volume of saline on days 3, 5, and 7 after tumor inoculation. For the dose–response study, groups of 10 animals were treated with a dose (in 40 μl) of 2.5 μg, 5.0 μg, or 10 μg of LMB-7, saline, or the control sFv immunotoxin anti-Tac(Fv)-PE38 on days 3, 5, and 7 after tumor inoculation. At the time of treatment initiation, five animals were killed and their neuraxes were processed for histology to approximate the tumor burden at the time of treatment. The animals were followed with daily weight and neurologic checks until death. At the time of death, each animal was given a complete autopsy. The neuraxis and liver were processed for histology, along with any organs with gross pathology.

Toxicity Studies. Nontumor-bearing rats were given an intrathecal dose of LMB-7 (10 μg in 40 μl) every other day for a total of three doses, a dose schedule identical to that used in the treatment studies. The animals were followed for 50 days with daily weight and neurologic function tests.

Histology. The neuraxis was processed as described (6). Sections were taken from the brain at the level of the coronal

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Abbreviation: mAb, monoclonal antibody.

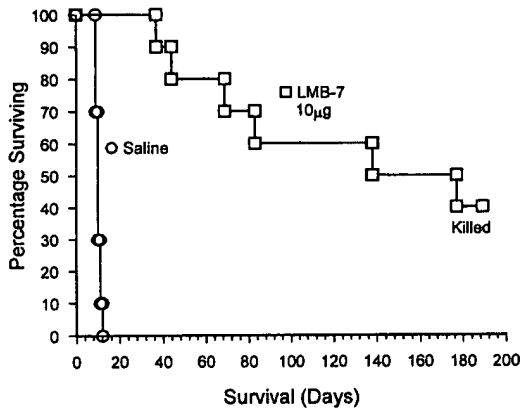

FIG. 1. Treatment of neoplastic meningitis from the human epidermoid carcinoma A431 in rats. The animals were treated with a dose (in 40 μl) of 10 μg of LMB-7 on days 3, 5, and 7 after tumor inoculation. LMB-7 increased median survival 1490% versus saline ($P < 0.001$).

suture and the pituitary gland and from the spinal cord at the cervical, thoracic, and lumbar levels and the cauda equina and stained with Luxol fast blue and with hematoxylin/eosin.

Statistical Methods. Survival cures were estimated for each treatment group by using the product-limit estimator of Kaplan and Meier (8). Comparison of these curves were conducted by using the log rank test (9).

RESULTS

To test the efficacy of LMB-7 on neoplastic meningitis, 5×10^6 A431 cells were introduced into each animal by using a preimplanted subarachnoid catheter. A431 cells were chosen for this study because they are derived from an epidermoid carcinoma that expresses large amounts of the B3 antigen. In cell culture, LMB-7 is very cytotoxic to A431 cells with an IC_{50} of 0.3–0.8 ng/ml. Furthermore, LMB-7 produces complete regressions of A431 cells growing as subcutaneous tumors in mice when three doses are administered intravenously, each at 0.063 mg/kg (1.25 μg per mouse) (6).

For the initial study, 10 μg of LMB-7 in 40 μl was administered to nude rats 3, 5, and 7 days after tumor implantation. The 10-μg dose was chosen because it produced no toxicity as manifested by death or loss of antigravity strength when administered to nontumor bearing rats; these animals were followed for 50 days before being sacrificed. The results of the first experiment in which LMB-7 (10 μg) or saline (diluent) were given to tumor bearing rats ($n = 10$) are shown in Fig. 1. All 10 rats that received saline on days 3, 5, and 7 died between days 7 and 12 (median, day 10), with a loss of antigravity strength preceding death (median, day 8.5). In contrast, the 10 rats treated with 10 μg of LMB-7 showed a dramatic response with the median survival extended to day 159. Furthermore, 4 of 10 rats survived to the end of the experiment at day 190. Autopsies showed that 2 of these long-term survivors were tumor-free, whereas 2 others had microscopic evidence of tumor in the cerebellum.

Next, a dose–response study was carried out by administering three doses of 10, 5, and 2.5 μg given 3, 5, and 7 days after tumor implantation (Fig. 2). Ten micrograms was the maximum dose that could be given since the protein concentration of LMB-7 was 0.25 mg/ml and a maximum volume of 40 μl could be administered. The control animals received either saline (the diluent for LMB-7) or anti-Tac(Fv)-PE38, a recombinant immunotoxin directed at the p55 subunit of the human interleukin 2 receptor, which is not present on A431 cells (10). As shown in Fig. 2, all three doses of LMB-7 produced significant antitumor effects. In this experiment, 10 μg produced an even more dramatic effect than in the first

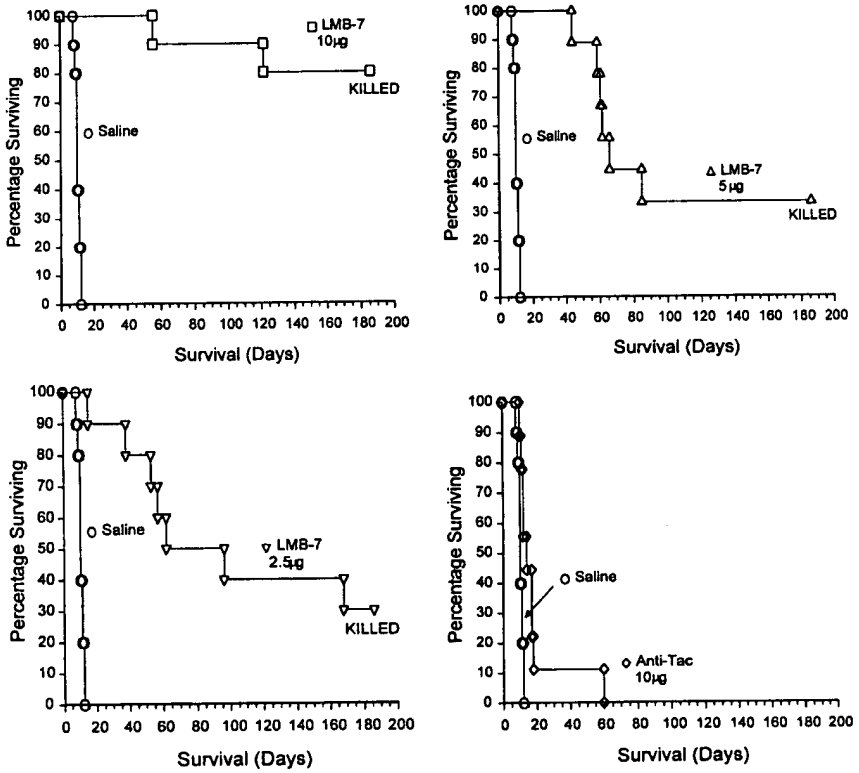

FIG. 2. Dose–response treatment of neoplastic meningitis from the human epidermoid carcinoma A431 in rats. The animals were treated with a dose (in 40 μl) of 10 μg (*Upper Left*), 5.0 μg (*Upper Right*), or 2.5 μg (*Lower Left*) of LMB-7 or 10 μg of anti-Tac(Fv)-PE38 (*Lower Right*) on days 3, 5, and 7 after tumor inoculation. Median survival was increased a minimum of 1807% (10 μg), 560% (5 μg), and 690% (2.5 μg) for the three LMB-7 dosage groups ($P < 0.001$ for all groups). Anti-Tac(Fv)-PE38 increased survival 40% compared with saline ($P = 0.032$).

experiment with 8 of 10 rats surviving until day 186, when the experiment was terminated. Only two animals died before day 186, one on day 56 and the other on day 122, and their deaths were preceded by loss of antigravity strength on days 39 and 77. By using the day the animals were sacrificed as the median survival, an estimate of the lower limit of median survival is 1807%. In the group treated with 5 μg of LMB-7, one animal died from an anesthesia death while receiving the third dose of immunotoxin. Six animals died between days 44 and 85 (median, 66 days), whereas the three other animals survived until the experiment was terminated. In the group treated with 2.5 μg of LMB-7 (n = 10), animals died between days 15 and 168 with three long-term survivors (median, 79 days). All the saline-treated animals were dead by day 12 (median survival, 10 days). In addition, a group of animals was treated with anti-Tac(Fv)-PE38, an immunotoxin directed at the p55 subunit of the interleukin 2 receptor (10). Anti-Tac(Fv)-PE38 is identical to LMB-7 except that the Fv portion binds to the p55 subunit of the interleukin 2 receptor, which is not expressed on A431 cells. When tested against cultured cells, anti-Tac(Fv)-PE38 is 500-fold less toxic to A431 cells than LMB-7 [B3(Fv)-PE38]. In the group treated with anti-Tac(Fv)-PE38, one animal died of anesthesia while receiving the second dose of immunotoxin (n = 9). The median survival of the other animals was 14 days with no long-term survivors.

When the median survival of the LMB-7-treated groups was compared with the saline controls, the percentage of increase was 1807% for 10 μg, 560% for 5.0 μg, and 690% for 2.5 μg. For the group treated with three 10-μg doses of LMB-7, the end of the experiment (day 186) was taken as the median day of survival. By the nonparametric log rank test, the survival of all LMB-7 treatment groups was statistically significant compared with saline controls ($P < 0.001$). The difference between the group treated with 10 μg of LMB-7 and the groups treated with 5 μg and 2.5 μg of LMB-7 was statistically significant ($P = 0.039$ and $P = 0.022$, respectively). No statistical significance was found in the difference between the groups treated with 5 μg and 2.5 μg of LMB-7.

Histologic analysis was performed on the neuraxes of all animals that died during an experiment. In almost all cases, a tumor was detected and was frequently accompanied by peripheral demyelination, edema, and hemorrhage. In a few animals, tumor was not detected but since all portions of the neuraxis were not sectioned we assume tumor was present in those locations. In most of the animals surviving to the end of the experiment (190 or 186 days), a tumor was not detected, but a tumor was present in a few of these animals. When given intravenously, the LD_{50} of LMB-7 is 7 μg per mouse (0.35 mg/kg) for three doses, and death is due to liver toxicity (L.H.P., unpublished data). No liver-related deaths were observed in mice given 10 μg per mouse for three doses by the intrathecal route. This is presumably because LMB-7 leaves the intrathecal space slowly and does not achieve high levels in the blood.

DISCUSSION

In this study, we have shown that neoplastic meningitis in athymic rats can be successfully treated with a recombinant single-chain immunotoxin (LMB-7) directed at an antigen present on many human cancers that metastasize to the brain but not present in normal human neural tissues. To carry out these studies, human epidermoid carcinoma cells were introduced into the intrathecal space and allowed to grow for 3 days before therapy was initiated. Untreated animals had a median survival of 10 days whereas in 8 of 10 animals in one experiment and 4 of 10 animals in another experiment that were treated with three doses of LMB-7 survived >180 days, when the experiment was terminated. The effect of LMB-7 [B3(Fv)-PE38] was specific since anti-Tac(Fv)-PE38, a recombinant immunotoxin directed at an antigen not present on the tumor, gave a very small increase in survival (median, 14 days).

The theoretical potential of specific immunotherapy with mAbs has been tempered by practical limitations. First, only a few tumor-specific surface antigens have been identified that can be used as specific tumor targets; one such antigen is the in-frame mutation of the epidermal growth factor receptor (11). A second obstacle to mAb therapy is poor delivery to the tumor. Poor delivery can be attributed to physiological barriers that exist within tumors: heterogeneous blood supply, elevated interstitial pressure, and large transport distances in the interstitium (12, 13). Numerous attempts have been made to alter the physiologic parameters of tumors to increase the delivery of macromolecules such as mAbs. Hyperthermia, radiation, and vasoactive drugs have been investigated as potential means of increasing blood flow (12, 14). To counteract the elevated interstitial pressure of tumors, osmotic agents that increase the vascular osmotic pressure have been studied (15). Another approach has been to increase the interstitial transport rate of molecules. This can be accomplished by using mAb fragments such as $F(ab')_2$, Fab, or small single-chain antigen-binding proteins that are produced by linking the variable regions of the light and heavy chains with a flexible linker (16, 17) or by a disulfide bond (18). A further way to approach the problems of the physiologic barriers encountered in tumors replaces commonly used systemic administrations with regional or compartmental delivery. Compartmental therapies with the mAbs that have been investigated include intraperitoneal injection, direct injection into cyst cavities within tumors, and intrathecal injection for the treatment of neoplastic meningitis (19, 20). Instilling mAbs directly into the intrathecal space can limit systemic exposure of noncentral nervous system tissues that share antigens with tumors targeted by the mAbs. This method can also eliminate the problem of physiologic barriers, leaving only rate of mAb diffusion as the major limiting factor in delivery of the therapeutic agent.

In this study, we investigated the therapeutic efficacy of the single-chain immunotoxin LMB-7 [B3(Fv)-PE38] in the treatment of human neoplastic meningitis in athymic rats. This model is directly applicable to the human because humans and rats share a subset of B3-positive normal tissues. The positive rat tissues include stomach, salivary glands, lungs, and pars intermedia of the pituitary (D.D.B. *et al.*, unpublished data). We have studied the treatment of neoplastic meningitis in athymic rats with LMB-1, a high molecular weight conventional immunotoxin. LMB-1 is composed of mAb B3 chemically linked to the PE38-engineered form of *Pseudomonas* exotoxin (D.D.B. *et al.*, unpublished data). In this model, three 200-μg doses of LMB-1 (same dose regimen as this study) produced an increase in median survival of 274% compared with saline controls. On a molar basis, the dose of LMB-1 (1.05 nmol) is approximately seven times that of LMB-7 (0.15 nmol), yet LMB-7 was much more effective. Several explanations are possible for the greater efficacy of the LMB-7 immunotoxin. One difference between LMB-1 and LMB-7 is their molecular size. LMB-1 is composed of an intact IgG chemically attached to a genetically engineered form of *Pseudomonas* toxin and has a molecular mass of ≈190 kDa. In contrast, LMB-7 is composed of only the heavy and light chain variable regions of mAb B3 fused to the same toxin and has a molecular mass of only 65 kDa. In tissue culture studies using the A431 cell line, both LMB-1 and LMB-7 have approximately equal activities (IC_{50} ≈10 pM). The primary reason for the greater efficacy of LMB-7 in the animal studies is most likely due to its smaller size relative to LMB-1. By using compartmental administration, we essentially bypass the limiting distribution factors associated with systemic administration. If no bulky disease exists at the time of treatment and, therefore, uniform flow of cerebrospinal fluid is present throughout the intrathecal

space, the limiting factor of therapy in these two models is diffusion. The formula for the amount of time required for molecular diffusion into tissue is $l^2/4D$, where l is distance of diffusion and D is diffusion coefficient, which is dependent on molecular size and shape (13). By using this equation, IgG would take ≈60 min to reach a distance of 100 μm, while $F(ab')_2$ (12) would take only 21 min to reach this distance. The size of the LMB-7 immunotoxin is on the order of that of $F(ab')_2$ fragments. In contrast, LMB-1 is considerably larger and more asymmetric than an IgG. Smaller molecules penetrate tumor tissue much faster than do larger ones and are, therefore, able to penetrate deeper into the multicellular layers of tumor.

The treatment of neoplastic meningitis can roughly be compared to the incubation of tumor spheroids bathed in solutions of mAbs. Sutherland *et al.* (21) studied the penetration of two different mAbs to carcinoembryonic antigen and the penetration of their $F(ab')_2$ and Fab fragments into tumor spheroids. Their studies showed that, for both of the intact anti-carcinoembryonic antigen mAbs, there was heterogeneous labeling from one to three cells deep after a 4-h incubation. Both $F(ab')_2$ and Fab fragments penetrated deeper than intact mAb, with Fab fragments penetrating deeper than $F(ab')_2$. Yokota *et al.* (22) studied the distribution of four immunoglobulin forms of the second generation mAb CC49, which reacts with the TAG-72 antigen, and found that, after 6 h, the IgG had penetrated the tumor to a depth of 40 μm, the Fab had penetrated to 70 μm, and the sFv had penetrated to 100 μm.

A second factor that must be considered is the influence of affinity on penetration (23). The K_d of LMB-7 is ≈1300 nM compared with 280 nM for LMB-1 (K. Webber, L.H.P., and I.H.P., unpublished data). Demignot *et al.* (24) suggested that the higher the affinity the greater the hindrance to penetration. The lower affinity of LMB-7 would, then, contribute to a deeper penetration and presumably, a greater reduction in the tumor mass. Baxter *et al.* (25) describes a two-pore model system that takes into account the influence of specific binding of mAb localization. This is supported by Milenic *et al.* (26) in the comparison of four immunoglobulin forms of the mAb CC49. The investigators found that the affinity of the Fab' and the sFv were 8-fold lower than the two dimeric forms, while the penetration of the sFv was greater than twice that of the intact IgG.

Increased therapeutic efficacy has been observed with other sFv immunotoxins as compared with their IgG chemical conjugate parent. The immunotoxin BR96 sFv-PE40 has shown the ability to totally regress subcutaneously growing L2987 lung carcinoma xenografts, whereas the IgG chemical conjugate could only keep the tumor size static (27). BR96 sFv-PE40 was found to inhibit *in vitro* protein synthesis four times better than the chemical IgG conjugate. In this instance, the greater efficacy is most likely due to a combination of immunotoxin size and increased ability to inhibit protein synthesis.

To our knowledge, there are currently no satisfactory methods of treating neoplastic meningitis due to the metastasis of solid tumors. Experiments using 4-hydroperoxycyclophosphamide, ACNU, melphalan, and ^{211}At-labeled antibody (81C6) (28–30) have produced increases in survival ranging from 41 to 111% in animals. Also, immunotoxins containing *Pseudomonas* exotoxin directed at a human small cell lung carcinoma have been evaluated and produced 35–40% increases in survival when given 1 day after tumor administration (31). These modest responses should be contrasted with the >1800% increase in survival noted in the current study using established tumors.

In summary, treatment with single-chain immunotoxin LMB-7 has resulted in long-term survival of rats with neoplastic meningitis produced by a human epidermoid carcinoma. These results suggest that this agent should be evaluated in humans with this disease.

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Note Added in Proof. Since submission of this manuscript, we have continued to evaluate the maximum tolerated dose of LMB-7 in non-tumor-bearing athymic rats. Groups of 12 non-tumor-bearing athymic rats were treated intrathecally with 40 μl of saline or saline containing 10, 15, and 20 μg of LMB-7 given every other day for a total of three doses. Animals treated with saline and 10 μg of LMB-7 showed weight gain and no loss of stepping and placing reflex or ability to climb an incline ramp. Toxicity was observed in the groups treated with 15 and 20 μg of LMB-7. Loss of stepping and placing reflex and ramp climbing ability was seen in 3 of 12 animals receiving 15 μg and 5 of 12 animals receiving 20 μg of LMB-7. In the 15-μg group, 3 of 12 animals died, and in the 20-μg group, 2 of 12 animals died. At 42 days, a predetermined group of 6 animals was killed from each dose level for investigation of acute toxicity. The remaining animals will be followed for a total of 180 days to determine chronic toxicity. Autopsy of the acute toxicity animals and of the 5 treated animals that died revealed no gross pathologic abnormalities, especially no neurotoxicity was observed. Histologic examination of the neuraxis and internal organs has not yet been done. Our data shows that a 10-μg intrathecal dose given three times every other day is the maximum tolerated dose that produces no toxicity in normal athymic rats.

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